The Patent Office

PCT/GB 96/02405

PCT/GB96/02405

The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation and Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

PRIORITY DOCUMENT

Signed

Dated 16 OCT 1996

Patents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

1/77

01OCT95 E145997-19 002823
P0177700 25.00

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

THE PATENT OFFICE
28 SEP 1995
LONDON

The Patent Office
Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

SJK/BP5193479

2. Patent application number
(The Patent Office will fill in this part)

28 SEP 1995 **9519776.0**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

CASIMIR Colin
c/o Institute of Child Health
University of London
30 Guilford Street
London WC1N 1EH

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

06856500GB

MATERIALS AND METHODS RELATING TO THE TRANSFER OF NUCLEIC ACID INTO STEM CELLS

5. Name of your agent *(if you have one)*

MEWBURN ELLIS

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

York House
23 Kingsway
London
WC2B 6HP

Patents ADP number *(if you know it)*

109006

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

No

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	15
Claim(s)	
Abstract	
Drawing(s)	2

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Mewburn Ellis Date 27/09/95

12. Name and daytime telephone number of person to contact in the United Kingdom Simon Kiddle Tel: 0117 9266411

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Materials and Methods Relating to the Transfer of Nucleic Acid into Stem Cells

Field of the Invention

The present invention relates to materials and methods relating to the transfer of nucleic acid into stem cells, and in particular to a method for stably transducing stem cells with nucleic acid encoding a desired protein or polypeptide so that the nucleic acid is incorporated into the genome of the stem cells. The present invention also relates to uses of this method, eg for gene therapy, and compositions for use in the treatment of various conditions.

Background to the Invention

The development of somatic gene therapy as a treatment for single gene inherited diseases and some acquired conditions, such as certain types of cancer, will represent one of the most important technical advances in medicine. Blood related disorders such as the X-linked immunodeficiencies, or chronic granulomatous disease (CGD), are amongst the most favourable candidates as model systems for the evolution of this technology. The general feasibility of gene therapy for disorders of this type has been amply demonstrated by the results obtained in treating adenosine deaminase dependent severe combined immunodeficiency (ADA-SCID) by gene transfer into peripheral blood T-cells.

However, many problems stand in the way of the realisation of the promise of these techniques. Thus, in the experiments described above, the T-cells are not immortal, requiring the therapy to be repeated at regular intervals. Further, attempts to effect a permanent correction, for example by gene transfer into pluripotent haematopoietic

stem cells (PHSC), have thus far been unsuccessful.

There are a number reasons for this. Firstly, PHSC are very rare in the bone marrow cell population, and so although work has been done on bone marrow cell culture, it is very difficult to draw conclusions from this work regarding PHSCs.

Further, in humans there is a dearth of markers to identify PHSC and, at present, the most reliable marker of immature human bone marrow cells is the CD34 antigen, which marks about 1-2% of total marrow cells. However, probably only about 0.1% of these CD34+ cells are true PHSC. In addition, there are no wholly reliable assays for human PHSC, unlike murine systems, where the rescue of lethally irradiated individuals can be used to test for PHSC.

Recently, however, a method to enrich for PHSC has been described by Beradi et al in Science, 267,104-108 (1995), which exploits the quiescence of PHSCs as a basis for their functional isolation. In this method, bone marrow cells were incubated for 7 days in the presence of the cytokines stem cell factor (SCF) and IL-3, to stimulate division in all of the progenitor cells, but not in true PHSC. The cytotoxic agent, 5-fluorouracil (5-FU), was then added to these cultures, resulting in the death of all dividing cells in the culture. However, quiescent cells, including PHSC which average only 1 in 10^5 of the original cells, were spared in this process. Accordingly, the authors reported obtaining an enriched population of cells having the characteristics of true PHSC.

However, the authors of this paper were unable to find any combination of cytokines that was able to stimulate these cells to divide, other than incubation in long term marrow culture (LTC), which also leads to their differentiation. Thus, although, this method can now produce highly enriched

populations of PHSC, it is their quiescence, the very property exploited for their isolation, that still represents the most significant hurdle limiting current gene therapy protocols. This is because most highly developed vector systems presently used for gene transduction are based on murine retroviruses and these viruses (and the vectors derived from them) are unable to stably integrate their genome into non-dividing cells.

Previously, we presented an abstract at the European Working Group for Gene Therapy in November 1994 disclosing that a retroviral cell line containing a viral vector incorporating nucleic acid encoding GCD and expressing stem cell factor on its surface was able to achieve improved rates of transduction in a bone marrow cell culture. However, as mentioned above this cell culture contains a very low proportion of PHSC, and this treatment would not be expected to stimulate the PHSC to divide or to allow the stable integration of the nucleic acid encoding GCD into the PHSC genome. An important fact underlying this expectation is that in Beradi et al, stem cell factor was one of the cytokines used to stimulate selectively division in the most of the cells in marrow cell culture (but not the PHSC), allowing them to be killed to leave the enriched population of stem cells.

In addition, there remains in the art a further major obstacle to using PHSCs as targets for gene therapy techniques which arises from the observation that PHSC are normally quiescent, in a stable G_0 state, which renders them refractory to retroviral gene transfer.

Summary of the Invention

The present invention is based on the unexpected finding that it is possible to get PHSC to cycle transiently during the period of exposure to vectors incorporating nucleic

acid encoding a desired protein or polypeptide by exposing them to bound stem cell factor. This observation means that, contrary to prior expectations, a population of PHSC can be used as targets for vectors (eg retroviral vectors) incorporating nucleic acid encoding a desired protein or polypeptide, provided that the PHSC are additionally exposed to bound growth factor, eg stem cell factor expressed by a retroviral packaging cell line so that it is bound on the cell surface or, alternatively, expressed on the surface of a retrovirus, eg as part of the retroviral envelope protein.

Broadly, the present invention provides a method for transferring nucleic acid encoding a desired protein or polypeptide to stem cells, the nucleic acid being incorporated into a vector, eg a viral vector, wherein the vector is capable of transfecting a population of dividing stem cells, resulting in the nucleic acid being stably integrated into the genome of the stem cells. This method has the advantage that it can be adapted for the treatment of a wide variety of disorders, by incorporating nucleic acid encoding the appropriate protein or polypeptide into the vector.

Accordingly, in one aspect, the present invention provides a retroviral packaging cell line containing a viral vector, the viral vector having a site for the insertion of nucleic acid encoding a desired protein or polypeptide, wherein the retroviral packaging cell line expresses a surface bound growth factor that is capable of stimulating the stem cells to divide. Thus, when nucleic acid has been incorporated into the viral vector, the cell line can be used to transduce the nucleic acid into stem cells.

In a further aspect, the present invention provides a retroviral vector incorporating nucleic acid encoding a desired protein or polypeptide wherein the retroviral

vector expresses a surface bound growth factor that is capable of stimulating the stem cells to divide.

In a further aspect, the present invention provides a method of transfecting nucleic acid encoding a desired protein or polypeptide into the genome of stem cells comprising exposing the stem cells to a vector incorporating the nucleic acid, wherein the stem cells are additionally exposed to a surface bound growth factor that is capable of stimulating the stem cells to divide.

Conveniently, the growth factor is provided by engineering the retroviral packaging cell line to express growth factor on its surface by transfecting the cell line with nucleic acid encoding the growth factor. In an alternative embodiment, a retroviral vector expressing surface bound growth factor (eg SCF) could be prepared by constructing a packaging cell line engineered to produce a chimeric retroviral envelope protein fused to all or part of the growth factor. The growth factor can be used to replace the natural binding domain of the envelope protein, or can be fused directly to the C-terminus. Such chimeric envelopes have been described for use in retroviral targeting (7-9). The chimeric envelope could be expressed as the sole viral envelope protein in an attempt to target the retrovirus to stem cells, as well as to transduce a growth signal, or in concert with the "wild type" envelope protein, to induce growth in growth factor responsive target cells, without targeting to a specific cell type. The former strategy is more applicable to an *in vivo* situation, the latter to an *in vitro* transduction process.

Preferably, the surface bound growth factor is stem cell factor, also known as mast cell growth factor, kit ligand factor or Steel factor. Nucleic acid sequences encoding stem cell factors are described in WO92/00376, eg the Δ28 MGF stem cell factor.

Preferably, the vector is a retroviral vector such as MFG or the pBabe vector series. Other vectors suitable for use in the methods described herein can be readily identified by the skilled person.

Typically, the desired protein or polypeptide will be one that a patient is unable to synthesise in his or her body or does not synthesise in the usual amount. However, the concepts described herein are applicable to situations in which the nucleic acid encodes a protein or polypeptide that binds a substance that is overexpressed in a patient's body, eg causing some harmful physiological effect, or a protein or polypeptide that can bind to a polypeptide that is produced in a patient's body in an inactive form to activate it or in an active form to inactivate it. The use of the present invention in these applications can have the advantage that the therapy provided by transfecting the stem cells is long lasting or permanent, thereby helping to avoid the need for frequently repeated treatment. Thus, the materials and methods described above can be used in the treatment of cancer or heart disease.

In a further aspect, the present invention provides stem cells as obtainable using the above method, that is stem cells having a nucleic acid encoding a desired protein or polypeptide stably incorporated into their genome.

In a further aspect, the present invention provides compositions comprising the above retroviral packaging cell line or retroviral vectors in admixture with a suitable carrier. In this aspect, the present invention provides pharmaceutical compositions suitable for delivering nucleic acid encoding a desired polypeptide to a population of stem cells *in vitro*, eg to prepare engineered stem cells for subsequent implant into a patient. Alternatively, the composition could be used *in vivo*, to directly deliver the nucleic acid to a patient's own stem cells. In this case,

the composition preferably comprises a retroviral vector incorporating the nucleic acid encoding a desired protein or polypeptide and displaying a growth factor on its surface, eg as part of an envelope protein.

In a further aspect, the present invention provides pharmaceutical compositions comprising the stem cells as obtainable using the above methods. In this aspect, the stem cells could be engineered *in vitro* and then implanted into patients in need of gene therapy.

In a further aspect, the present invention provides the above pharmaceutical compositions for use in methods of medical treatment, especially in gene therapy.

In a further aspect, the present invention provides the above retroviral packaging cell line or retroviral vecotors for use in the preparation of a medicament for transducing nucleic acid encoding a desired protein or polypeptide into the genome of stem cells.

In a further aspect, the present invention provides the above stem cells containing nucleic acid encoding a desired polypeptide for use in the preparation of a medicament for treating a condition that responds to the desired protein or polypeptide.

Detailed Description

Brief Description of the Figures

Figure 1. A) top panels, bone marrow cells following 5 days incubation with 5-FU (right) or without 5-FU (left); bottom panels, staining of cells as above for SCF receptor at completion of 7 days selection in 5-FU (right) or without 5-FU (left). B) PCR analysis of colonies arising from retrovirally transduced, 5-FU selected, stem cells in semi-

solid medium following 4 weeks long term culture. 1-9, colonies; N, negative control; C, positive control; M, size markers. The arrow indicates the retroviral PCR product.

Figure 2. Tritiated thymidine labelling of 5-FU selected cells. Bone marrow cells were incubated as described earlier for 7 days in 5-FU [A], or not [B], after which tritiated (^{3}H) Thymidine was added to the medium and the cells incubated for a further 16 hrs. Following this incubation they were pelleted onto glass microscope slides using Cytospin (Shandon Instruments). The slides were dipped in photographic emulsion (Ilford) and allowed to dry before incubation in the dark at -70°C for one week. The slides were then developed using standard developer and fixer and counter stained with Wright's stain. Cells undergoing division are labelled by the incorporation of ^{3}H thymidine into DNA, which leads to the formation of silver grains in the emulsion. The 5-FU treated cells (panel A) show no labelling indicating quiescence, whereas the untreated cells (panel B) show extensive and intense labelling indicative of active cell division.

Materials and Methods

Production of the retroviral packaging cell line

The cell line 1MI-ΔSCF was constructed as follows: the parent producer cell line 1MI was derived from the Am12 packaging cell line (1), by calcium phosphate-mediated DNA transfection, using the retroviral vector encoding the p47-*phox* cDNA we described previously (2), with the exception that the neomycin resistance cassette was removed. The retroviral backbone is derived from the pBabe series of vectors described by Morgenstern et al (3). High titre producer clones were then selected by "dot blot" analysis of successful transfectants. The 1MI producer line was then transfected as described above using the plasmid pJP2

encoding the membrane-associated form of the human stem cell factor (SCF). Cells expressing SCF were selected using histidinol. Individual clones were grown out and tested for expression of SCF by immunofluorescence with a labelled anti-SCF antibody. The plasmid pJP2 was constructed by insertion of an 816bp HindIII to BamH1, SCF cDNA fragment into the mammalian cell expression plasmid pREP8 (Invitrogen Corp). The SCF cDNA was excised from the plasmid BSSK: huMGFΔ28, see WO92/00376.

Selection and transduction of PHSC

Bone marrow cells (10mls; approx $5x10^7$ cells) were aspirated from the iliac crest of normal volunteers under local anaesthesia. The cells were washed twice with sterile PBS, re-pelleted and layered onto the surface of a discontinuous ficoll gradient. Cells were separated by centrifugation for 20 mins at 2500 rpm. Mononuclear cells were removed from the interphase and washed with PBS. Cells were then incubated in Iscove's DMEM medium supplemented with 10 % fetal calf serum, 5-fluorouracil (5-FU), stem cell factor (SCF) and IL-3, as described by Beradi et al (Science 267 1995). Following seven days in selection (see Fig 1A), the surviving cells were co-cultured for 48hrs in the presence of the SCF-producer line. Following co-cultivation, they were removed from the producers and used to establish long term cultures (LTC) on heterologous irradiated human stroma, in McCoy's medium modified for long term culture. After 4 weeks in LTC, cells were plated in semi-solid media containing cytokines (StemGEM™), to allow colonies to develop.

Detection of transduced cells

Transduction was scored by PCR analysis of colonies (Fig 1B). The PCR relied on a nested strategy using two upstream and one downstream primers. An initial round of

35 cycles of amplification using the most upstream primer and the downstream primers was performed. A small aliquot of this reaction was removed and re-amplified in a second reaction using the second upstream primer and the downstream primer. The most upstream primer is complementary in sequence to a region from the *gag* gene of the retroviral vector and the other two primers are complementary to different regions from the p47-phox cDNA sequence. The size of the initial product is 454 nucleotides and the nested product 180 nucleotides. This strategy ensures that the PCR product is specific for the retrovirally encoded p47-phox gene and not the endogenous gene. The products of the PCR amplification were visualised under ultra-violet light (300nm) following separation by standard agarose gel electrophoresis on 2% gels containing ethidium bromide.

Results

The data set out in table 1 on two independent marrow cell cultures indicated that approximately 17% and 25% of colonies were positive for the presence of the retroviral genome (Table 1).

Table 1

	Total Colonies Assayed	Positive Colonies	%Positive Colonies
Expt 1 SCF producers	20	5	25
Expt 2 SCF producers	30	5	17
Non-SCF producers	30	0	0

Discussion

The above results show that good levels of transduction of PHSC can be achieved using engineered retroviral packaging cells expressing human SCF on their cell surface. Thus, the initial results above indicate that the cells should be capable of simultaneously delivering both a growth signal and a retroviral vector to the target PHSC. This simultaneous delivery of vector and growth signal should also have the advantage of increasing the effective retroviral titre, owing to the intimate association of producer and target cells.

SCF has been shown to have both soluble and membrane-bound forms. Evidence acquired from the study of mice carrying a small intragenic deletion in the gene encoding the SCF receptor has indicated that the membrane-bound form of the cytokine is essential for normal haematopoiesis. Despite being able to synthesise a soluble SCF retaining full biological activity, these mice are as badly affected as their counterparts who carry a complete deletion of the gene. While not wishing to be bound by any particular theory, we believe that the *in vivo* biological activities of the soluble and membrane-associated forms of the growth factor are distinct, and that normal haematopoiesis has an absolute requirement for the membrane-bound form of SCF that cannot be substituted by the soluble form. It may also be that the expression of bound SCF on the cell surface changes/reduces the extent to which other growth factors are expressed, and that this has a beneficial effect on transduction levels of the PHSC.

It may be possible to improve the transduction rates achieved in the claimed method using the synergistic action of additional cytokines. In this regard, SCF is particularly noted for its property of interacting in this

way with other growth factors, which has led to the suggestion that on its own it may not be a mitogen but acts as an anti-apoptotic factor. To assess this, similar experiments to those described above can be performed using additional cytokines added to the media in conjunction with our modified producers. Ideally, we would hope to find conditions favouring self-renewal at the expense of differentiation. This would have the highly desirable consequence of enabling us to expand PHSC numbers in culture. One factor thought possibly to act in this way is MIP1-α1. There is also evidence that stem cell quiescence may be negatively influenced by TGF-β, antagonists of this molecule may therefore be beneficial in stimulating cells into cycle. Of the positively acting cytokines, LIF, the factor that blocks differentiation of mouse embryonal stem cells and IL-11, a recently identified member of the same family of cytokines, are candidates for acting on stem cells, as is flt3 ligand, a molecule with a similar spectrum of activities to SCF.

The above method describes a protocol which is potentially applicable to any clinical procedure requiring the transfer of genetic information to pluripotent haematopoeitic stem cells (PHSC). As discussed above, this method is applicable for gene therapy of inherited haematopoeitic disorders, such as the immunodeficiencies, but it could also be applicable to conditions such as haemophilia, or other conditions requiring the synthesis of a pharmacologically active compound normally present in the serum. There are also potential applications in the field of cancer therapy, primarily as a way of protecting cells from cytotoxic agents or radioprotecting them, thus giving them a survival advantage over non-treated bone marrow cells.

Other diseases that might be treated using the above protocol include:

Chronic Granulomatous Disease (CGD), all forms.

Severe Combined Immunodeficiency (SCID), all forms.

Hyper gamma globulinaemia syndrome (Hyper IgM).

Wiskott-Aldrich Disease (WAS).

Thallassaemia, sickle-cell anaemia, other anaemias due to deficiencies of red blood cell proteins.

Neutrophil defects due to failure to synthesise granule components eg myeloperoxidase deficiency.

Haemophilia and other clotting disorders such as complement deficiencies.

Lysomal Storage Disorders, eg Gaucher's disease, Hurler's disease, Mucopolysaccharidosis.

Leukocyte Adhesion Deficiency (LAD).

Bare Lymphocyte Syndrome.

Cancer.

AIDS.

References:

1. Markowitz, D., S. Goff, and A. Bank. 1988. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* 167:400.

2. Thrasher, A., M. Chetty, C. Casimir, and A. W. Segal. 1992. Restoration of Superoxide Generation to a Chronic Granulomatous Disease-Derived B-Cell Line by Retrovirus Mediated Gene Transfer. *Blood* 80:1125.

3. Morgenstern, J. P. and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587.

4. Anderson, D. M., D. E. Williams, R. Tushinski, S. Gimpel, J. Eisenman, L. A. Cannizzaro, M. Aaronson, C. M. Croce, K. Huebner, and D. Cosman. 1991. Alternate splicing of mRNAs encoding human mast cell growth factor and localisation of the gene to chromosome 12q22-12q24. *Cell Growth Differ* 2:373.

5. Beradi, A. C., A. Wang, J. D. Levine, P. Lopez, and D. T. Scadden. 1995. Functional isolation and characterization of human hematopoietic stem cells. *Science* 267:104.

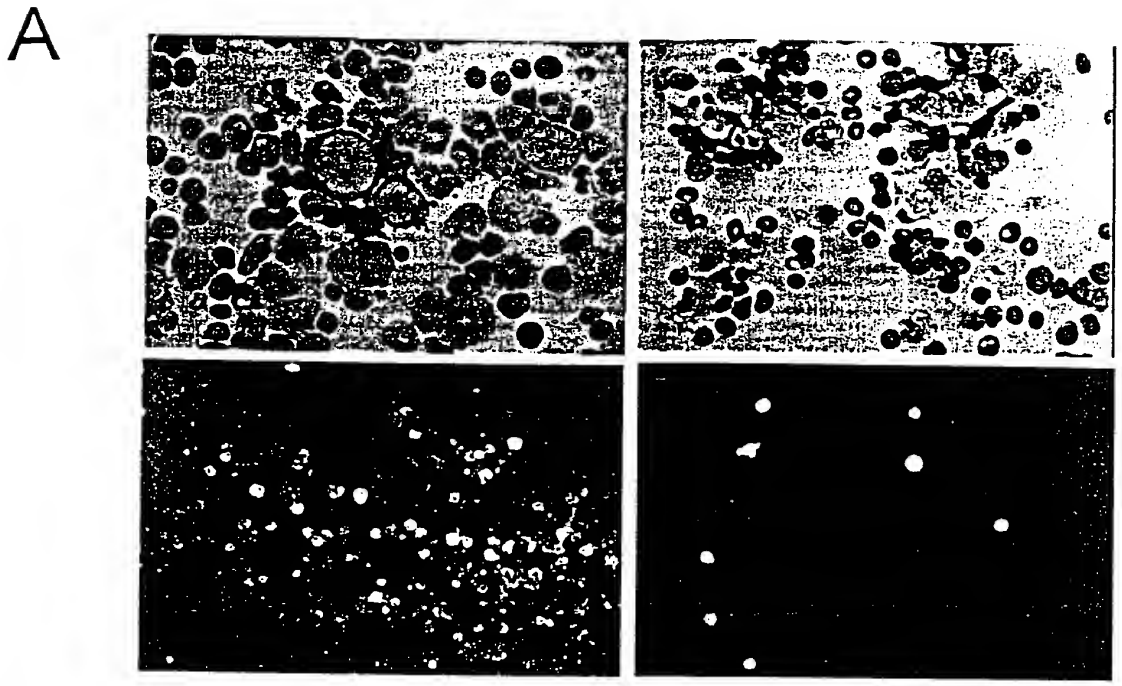
6. Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol* 51: 263.

7. Russell, S.J., R.E. Hawkins, and G. Winter, 1993. Retroviral vectors displaying functional antibody fragments.

8. Valesia-Wittman, S., A. Drynda, G. Delange, M. Aumailley, J.M. Heard, O. Danos, G. Verdier, and F.L. Cosset. 1994. Modifications in the binding domain of avian retrovirus envelope protein to redirect the host range of retroviral vectors. *J. Virol.*, 68:4609.

9. Kasahara, N., A.M. Dozy and Y.N. Kan. 1994. Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. *Science*, 266:1373.

Figure 1



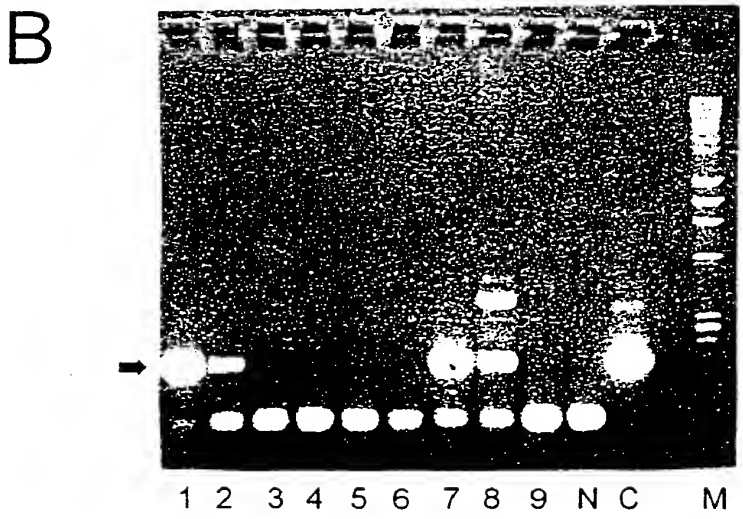


Fig 1. A) top panels, bone marrow cells following 5days incubation with 5-FU (right) or without 5-FU (left); bottom panels, staining of cells as above for SCF receptor at completion of 7 days selection in 5-FU (right) or without 5-FU (left). B) PCR analysis of colonies arising from retrovirally transduced, 5-FU selected, stem cells in semi-solid medium following 4 weeks long term culture. 1-9, colonies; N, negative control; C, positive control; M, size markers. The arrow indicates the retroviral PCR product.

Fig. 2

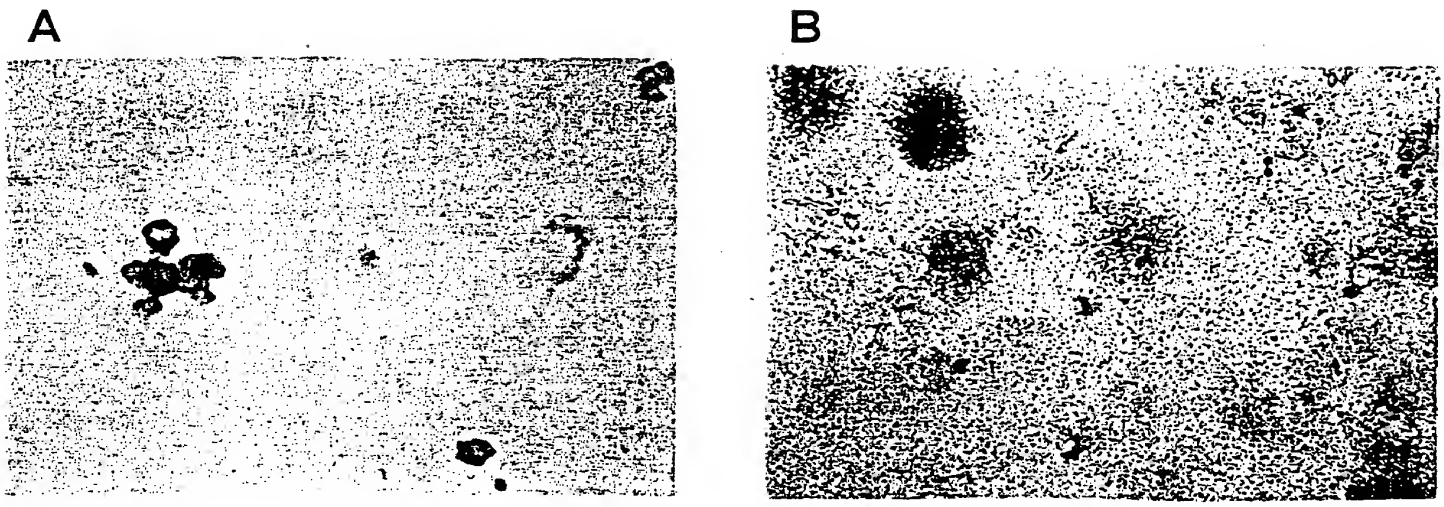


Figure 2. Tritiated Thymidine Labelling of 5FU Selected Cells

Bone marrow cells were incubated as described earlier for 7days in 5FU [A], or not [B], after which tritiated (^{3}H) Thymidine was added to the medium and the cells incubated for a further 16hrs. Following this incubation they were pelleted onto glass microscope slides using cytospin (Shandon Instruments). The slides were dipped in photographic emulsion (Ilford) and allowed to dry before incubation in the dark at -70°C for one week. The slides were then developed using standard developer and fixer and counter stained with Wright's stain. Cells undergoing division are labelled by the incorporation of ^{3}H Thymidine into DNA, which leads to the formation of silver grains in the emulsion.The 5FU treated cells (panel **A**) show no labelling indicating quiescence, whereas the untreated cells (panel **B**) show extensive and intense labelling indicative of active cell division.

PCT/GB96/02105

9519760

Mewburn Ellis

30.9.96